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DISEASE MODIFYING POTENTIAL OF WEDELOLACTONE RICH FRACTION OF *ECLIPTA ALBA* IN ADJUVANT INDUCED ARTHRITIS IN RATS BY INHIBITION OF PRO-INFLAMMATORY CYTOKINES

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ABSTRACT: *Eclipta alba* (Family-Asteraceae) is a herb commonly used in traditional Ayurvedic medicine for the treatment of inflammation, pain, and wounds. The present study is aimed to validate the ethnobotanical use of *Eclipta alba* in an animal model. The animals were induced with arthritis by injection of FCA on day 0 and treated with wedelolactone rich fractions of *Eclipta alba* (100, 200 and 400 mg/kg) from day 12 to day 28. WEA caused a significant effect in arthritis by inhibiting the joint inflammation and decreasing hyperalgesia and allodynia. WEA significantly decreased the biochemical markers and serum Tumor Necrosis factor-α, Interleukin 1β and Interleukin-6 levels and significantly increased the antioxidant profile. WEA (400 mg/kg) exhibited anti-rheumatic activity as evidenced by altered hematological milieu (ESR, CRP, WBC, RBC and Hb), histopathology of ankle joints, reduced cytokine levels, paw volume and related parameters associated with arthritis. Taken together, these results demonstrated the antiarthritic activity of WEA against experimental arthritis, and the underlying mechanism behind this efficacy might be mediated by inhibition of proinflammatory cytokines by wedelolactone in combination with other phytoconstituents.

INTRODUCTION: Rheumatoid arthritis (RA) is one of the prime health predicaments worldwide, which is the foremost cause of disability and the most common autoimmune disease in the world, leading to premature death if not treated properly [1]. In RA, inflammation of synovial tissue lining the joint capsule results in an invasion of the cartilage and bone, leading to progressive joint dysfunction manifested as synovitis, synovial hyperplasia, stiffness, and pain [2].

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The extent of inflammation is determined by the balance between proinflammatory and anti-inflammatory cytokines [3]. Reactive oxygen species, addition to cytokines, play a crucial role in the development and progression of RA [4]. Both sexes are affected while females are more susceptible to the ratio of 3:1. Conventional treatment with NSAIDs, DMARDs gives symptomatic relief, and newer biologicals like tumor necrosis factor-α (TNF-α) antagonist brought a therapeutic revolution by improving clinical, functional, and radiographic outcomes. However, the adverse effects, toxicity, and cost of the existing drugs appeal for a new alternative cost-effective therapy, which addresses the multiple targets in the treatment of RA [5].

Herbs have been in use from the time immemorable as a preventive and therapeutic medicine. Extensive

research is going on to scientifically validate the potential medicinal value of such plants [6]. India has a rich collection of medicinal plants distributed in different geographical and ecological conditions widespread throughout the country. These plants endow with active principles and are proven to be valuable in the treatment of various diseases, which lead many to study the phytoconstituents from plants [7, 8]. *Eclipta alba* (Linn.) Hassk, Family – Asteraceae is commonly known as "Bhringarajah". It is widely distributed throughout India, China, Thailand, and Brazil. The chemical constituents reported in the plant include wedelolactone, demethyl wedelolactone, thiophene-derivatives, steroids, triterpenes, luteolin, ecliptine and stigmasterol [9]. The herb is traditionally used to alleviate pain and inflammation [10] and reported to have anti-inflammatory [11], antinociceptive [12,] and nootropic [13] activities.

Wedelolactone is reported to suppress LPS-induced caspase-11 expression (a key regulator of cytokine IL-1β maturation) and pathological apoptosis [14]. Wedelolactone has also been reported to inhibit activation of NF-κB, production of NO, PGE2, TNF-α, inducible Nitric Oxide Synthase and cyclooxygenase-2 [15]. However, no attempts are reported to validate the antiarthritic potential of *Eclipta alba*. Considering the traditional analgesic and antiinflammatory use and *in-vitro* effects of its principle constituent wedelolactone, in present study, effort was made to enrich the extract with wedelolactone. The extract was standardized using high-performance liquid chromatography (HPLC) method, and wedelolactone rich fractions of *Eclipta alba* (WEA) were subjected to preclinical evaluation in Freund's Complete Adjuvant (FCA) induced arthritis.

MATERIALS AND METHODS:

Procurement and Authentication of Plant: *Eclipta alba* was collected in the month of January 2014, from Pune, Maharashtra, India. The plant was authenticated by the Botanical Survey of India, Pune and voucher specimen (No BSI/WRC/ Cert./2014/AS01) was deposited for future reference.

Drugs and Chemicals: FCA (Sigma Aldrich, USA), etoricoxib (gift sample from Zydus Cadila, Gujarat), Wedelolactone (Natural Remedies, India), biochemical diagnostic kits (Accurex Biomedical Pvt. Ltd.) were used. ELISA kits were procured from Ray Biotech Lexington, KY. All other solvents and chemicals used for the study were of analytical grade from authentic vendors.

Animals: Female Wistar rats (180–220g) were obtained from National Toxicology Centre, Pune, India. The animals were maintained at 25 ± 1 °C temperature and 45 to 55% relative humidity under 12 h light: 12 h dark cycle. The animals had free access to food pellets (Pranav Agro Industries Ltd, India) and water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA) (Registration No: 1703/PO/c/13/CPCSEA), India (Approval No CPCSEA/28/2014).

Extraction: The aerial part of the plant was shade dried, powdered, and was subjected to Soxhlet extraction by ethanol and filtered to get ethanolic extract. This ethanolic extract was concentrated and washed with hot water. This was fractionated with equal volume of ethyl acetate in separating funnel [16]. The collected ethyl acetate fraction was concentrated by rota evaporator (EquitorRoteva, Medica Instruments Mfg Ltd.) to get wedelolactone rich fraction of *Eclipta alba* (WEA).

Quantification of Wedelolactone using HPLC Method: The wedelolactone content in ethanolic extract and WEA was quantified using HPLC method. The solutions of ethanolic extract (1000 µg/ml), WEA (1000µg/ml) and wedelolactone (50 µg/ml) were prepared in methanol. The HPLC system (Jasco Corporation, Japan) was equipped with dual pump Jasco PU- 2080 Plus, UV/Visible detector UV-2075 plus; Thermo Scientific Merck C18 reversed-phase column (I.D. 4.6mm × 250mm, 5µm). The separation was achieved with a two-pump linear gradient program for pump A (water containing 0.1% glacial acetic acid) and pump B (acetonitrile) in the ratio of 40:60, respectively at 243 nm. Flow rate and injection volumes were 1.0 ml/min and 20µl, respectively. The chromato-graphic peak of the ethanolic extract and WEA was confirmed by comparing the retention time and UV spectra with the reference standard wedelolactone.

Acute Toxicity Studies: Acute toxicity studies were carried out for WEA following OECD guidelines No. 423 [17]. The WEA was suspended in 2% w/v Tween 80 and administered orally at the dose of 5 mg/kg body weight to overnight-fasted, healthy female rats (n=3).

The animals were observed individually for behavioral and autonomic profiles after dosing with special attention given during the first 4 h, and daily thereafter, for a total period of 14 days. The test was repeated with doses of 50, 300, and 2000 mg/kg body weight.

FCA Induced Arthritis: The animals were divided into six groups of six animals each. Group I served as healthy control (2% w/v Tween 80), Group II as arthritic control (2% w/v Tween 80), Group III as standard which received 10 mg/kg etoricoxib (p.o.), Group IV, V and VI received 100, 200 and 400mg/kg WEA (p.o.), respectively. All animals except healthy control groups were injected with 0.1 ml of FCA in the subplantar region of the left hind paw on day 0. The respective treatment started once signs of arthritis set in (day 12), orally once daily.

Bodyweight, paw volume, pain threshold, thermal and mechanical hyperalgesia, and tactile allodynia were evaluated on days 0, 1, 4, 8, 12, 16, 20, 24, and day 28. On day 28, blood was withdrawn by retro-orbital puncture under ether anesthesia for hematology; serum was separated for biochemical parameters and cytokine estimation [18]. The animals were sacrificed by CO_2 euthanasia, and spleen, thymus, liver, and ankle joints were isolated.

Body Weight: Body weight was recorded on all the above-mentioned evaluation days using animal weighing balance [4].

Paw Volume: Paw volume was measured using a Plethysmometer (UGO Basile, Italy). The change in paw volume was calculated as the difference between the final and initial paw volume [19].

Mechanical Hyperalgesia (Paw Withdrawal Threshold): It was measured as the paw withdrawal threshold of the animal in the Randall-Selitto analgesia meter (UGO Basile, Italy). The hind paw was placed between the flat surface and blunt pointer, and increasing pressure was applied. The pain threshold was determined when rat attempted to remove the hind paw from the apparatus. The cut-off pressure was 450g [18].

Thermal Hyperalgesia (Paw Withdrawal Latency): It was measured as paw withdrawal latency in radiant heat apparatus (UGO Basile, Italy). The paw was placed on the heat radiator with infrared intensity of lamp set at 40. A cut off latency of 15 sec was used to avoid tissue damage [18].

Tactile Allodynia (Mechanical Nociceptive Threshold): It was determined as a mechanical nociceptive threshold by measuring paw withdrawal upon probing of the plantar surface with a series of calibrated fine filaments (von Frey hairs, Almemo, Germany) of increasing gauge (0.6 to 12.6g). The rats were allowed to acclimatize for 10 min in the perspex box. A series of three stimuli were applied to each paw with each hair in a period of 2-3 sec. The lowest weight of von Frey hair to evoke a withdrawal from the three consecutive applications was considered as a threshold. Lifting of the paw was recorded as a positive response [18].

Measurement of Cytokine Levels: On day 28, serum Tumor necrosis factor-α (TNF-α), Interleukin 1β (IL-1β) and Interleukin-6 (IL-6) were determined using ELISA kit by sandwich method [18].

Hematological and Biochemical Parameters: On day 28, RBC count, hemoglobin (Hb), and platelet (PLT) count were determined by usual standardized laboratory methods [16]. Serum was used for the estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein (TP), and C-reactive protein (CRP) [18].

Spleen and Thymus Weight: On day 28, rats were sacrificed by CO_2 euthanasia. The spleen and thymus of all the rats were removed and weighed [18]. The liver was isolated for antioxidant studies, while the ankle joints were isolated for histopathology.

Antioxidant Parameters: The liver isolated after sacrificing was washed in ice-cold saline and homogenated with 0.1M Tris-HCl buffer (pH 7.4). The supernatant was used to determine superoxide dismutase (SOD) [19], malondialdehyde (MDA) [20], and reduced glutathione (GSH) [21].

Histopathological Analysis of Ankle Joints: The ankle joints separated from the hind paw were immersed in 10% buffered formalin and processed for paraffin embedding section at 5 µ thickness. The sections were stained with hematoxylin-eosin dye and evaluated under a light microscope with 10X magnifications [18].

Statistical Analysis: The data were analyzed by one way ANOVA followed by Dunnett's test for biochemical analysis, two way ANOVA followed by Bonferroni's post hoc test for *in-vivo* parameters. All statistical analyses were performed using Graph Pad Prism software (San Diego, CA). Data were considered statistically significant at $P<0.05$.

RESULTS:

Standardization of Extract: The yield of the plant extract was found to be 8.3% w/w. The HPLC analysis of the ethanolic extract and WEA confirmed the presence of wedelolactone (6.62% w/wand 25.3 % w/w, respectively) **Fig. 1A, 1B,** and **1C**.

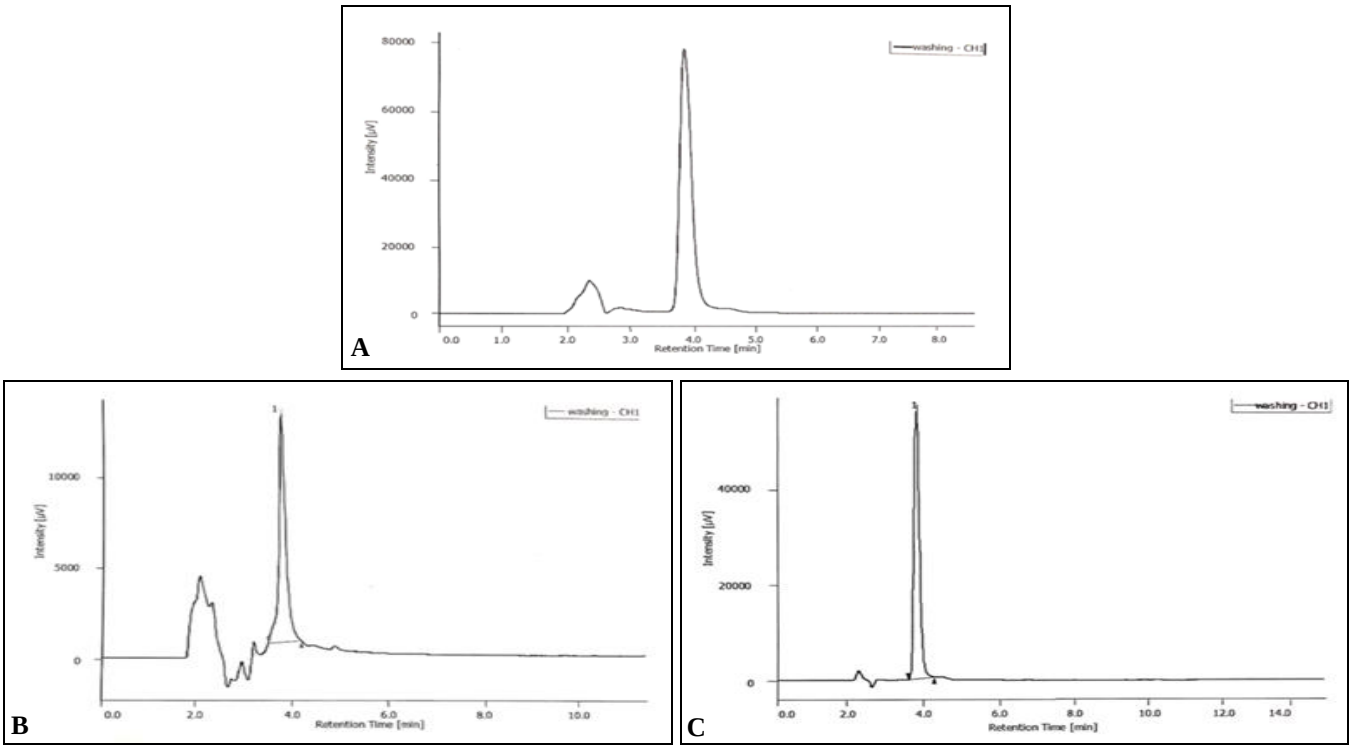

FIG. 1: CHROMATOGRAPH. A: WEDELOLACTONE STANDARD 50 PPM RETENTION TIME 3.4 min, B: EEA 1000 PPM RETENTION TIME 3.45 min, C: WEA 1000 PPM RETENTION TIME 3.45 min

Acute Toxicity Studies: The test animals did not exhibit any change in autonomic, behavioral profile and survived beyond the recommended duration of observation with 2000 mg/kg of WEA (OECD Guideline No. 423). Hence, it was safe up to 2000 mg/kg.

Effect of WEA on Body Weight in FCA Induced Arthritis in Rats: There was a significant decrease in body weight of all arthritic animals. On treatment with etoricoxib there was a non-significant increase in body weight when compared with arthritic control group of animals. Furthermore, treatment with WEA non-significantly improved the bodyweight when compared to arthritic control group **Fig. 2**.

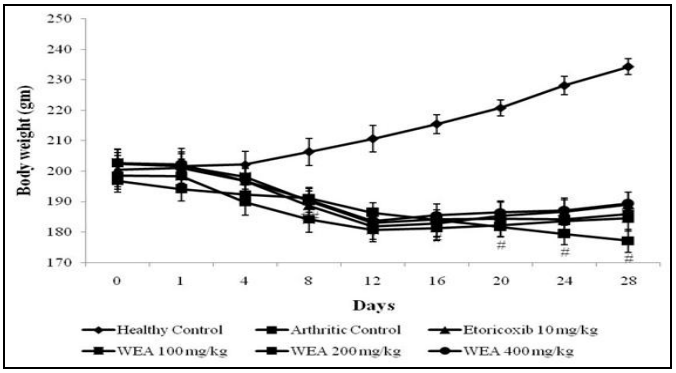

FIG. 2: EFFECT OF WEA ON BODY WEIGHT IN FCA INDUCED ARTHRITIS IN RATS. Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of WEA on Paw Volume in FCA Induced Arthritis in Rats: There was a significant increase in paw volume of all the rats in the arthritic control group when compared to healthy control. Treatment with etoricoxib and WEA (200 and 400 mg/kg) significantly decreased the paw volume from day 16 and 24 onwards, respectively as compared to arthritic control group. WEA at the dose of 100mg/kg was comparatively less effective and significantly decreased the paw volume on day 28 **Fig. 3**.

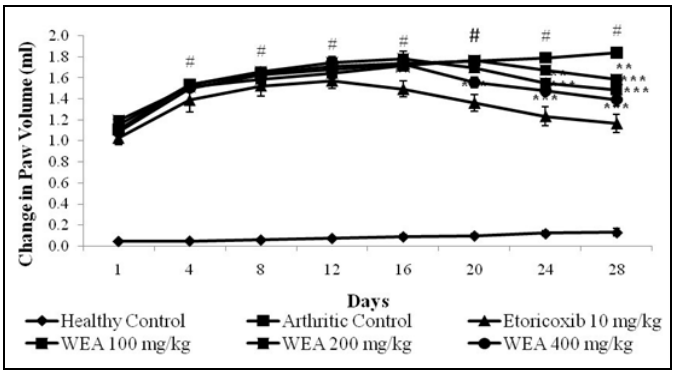

FIG. 3: EFFECT OF WEA ON PAW VOLUME IN FCA INDUCED ARTHRITIS IN RATS. Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. **P<0.01, ***P<0.001 when compared with Arthritic control and # P<0.001 when arthritic control compared to healthy control

Effect of WEA on Mechanical Hyperalgesia (Paw Withdrawal Threshold) in FCA Induced Arthritis in Rats: Paw withdrawal threshold in all arthritic animals decreased progressively till day 12 when compared to healthy control animals. The paw withdrawal threshold in arthritic control animals was significantly less compared to healthy control animals until the end of the study.

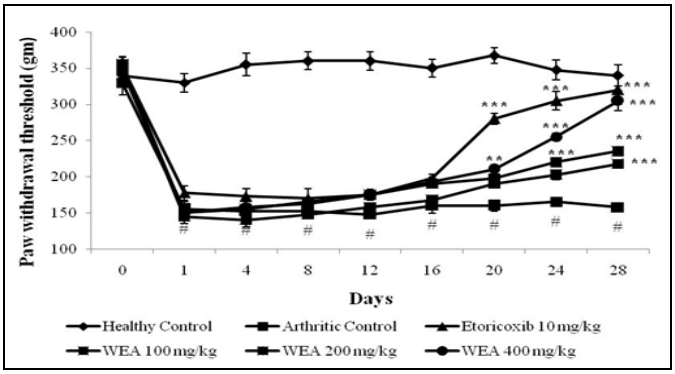

FIG. 4: EFFECT OF WEA ON MECHANICAL HYPERALGESIA (PAW WITHDRAWAL THRESHOLD) IN FCA INDUCED ARTHRITIS IN RATS. Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. **P<0.01, ***P<0.001 when compared with Arthritic control and # P<0.001 when arthritic control compared to healthy control.

Treatment with etoricoxib and WEA (400 and 200 mg/kg) significantly increased the paw withdrawal threshold from day 20 and 24 (8 and 12 days of treatment, respectively) onwards, respectively. However, WEA (100 mg/kg) significantly increased pain threshold only on day 28 (16 days of treatment) **Fig. 4**.

Effect of WEA Onthermal Hyperalgesia (Paw Withdrawal Latency) in FCA Induced Arthritis in Rats: On induction of arthritis, there was a significant decrease in paw withdrawal latency of all arthritic animals till day 12 when compared to healthy control **Fig. 5**. Treatment with etoricoxib and WEA (400 and 200 mg/kg) significantly increased the paw withdrawal latency from day 20. However, WEA 100 mg/kg significantly (p< 0.001) increased paw withdrawal latency only on day 28.

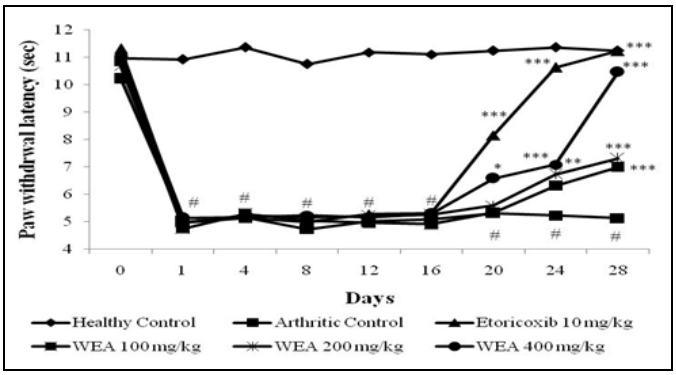

FIG. 5: EFFECT OF WEA ON THERMAL HYPERALGESIA (PAW WITHDRAWAL LATENCY) IN FCA INDUCED ARTHRITIS IN RATS. Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. *P<0.05, **P<0.01, ***P<0.001 when compared with Arthritic control and # P<0.001 when arthritic control compared to healthy control.

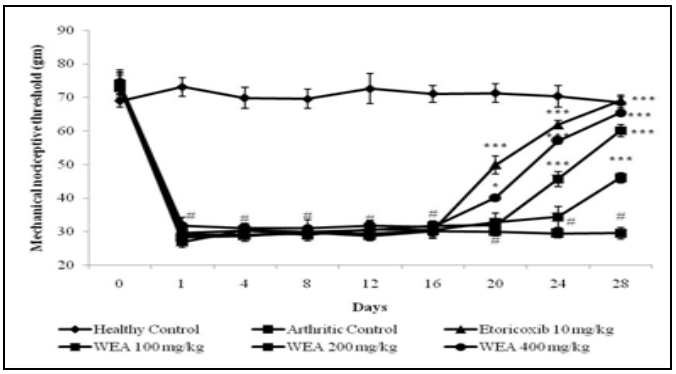

FIG. 6: EFFECT OF WEA ON TACTILE ALLODYNIA (MECHANICAL NOCICEPTIVE THRESHOLD) IN FCA INDUCED ARTHRITIC RATS. Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. *P<0.05, ***P<0.001 when compared with Arthritic control and # P<0.001 when arthritic control compared to healthy control.

Effect of WEA on Tactile Allodynia (Mechanical Nociceptive Threshold) in FCA Induced Arthritis in Rats: The mechanical nociceptive threshold was significantly decreased in arthritic animals till day 12. Administration of etoricoxib and WEA (200 and 400 mg/kg) from day 12 significantly improved the mechanical withdrawal threshold from day 20 onwards when compared to arthritic control while WEA 100 mg/kg significantly increased mechanical nociceptive threshold only on day 28 **Fig. 6**.

Effect of WEA on Hematology and Serum Parameters in FCA Induced Arthritis in Rats: There was a significant increase in platelet count, WBC count, and ESR while a decrease in RBC count and Hb level observed in the arthritic control group when compared to the healthy control group. These conditions reversed significantly and dose-dependently on treatment with WEA **Table 1**.

The serum CRP level was significantly increased in the arthritic control group as compared to the healthy control group. On treatment with etoricoxib and WEA (400 and 200 mg/kg), serum CRP level was found to be significantly decreased when compared with the arthritic control group **Table 1**.

The serum levels of AST, ALT and ALP were increased significantly in arthritic control group as compared to healthy control group. Treatment with etoricoxib and WEA (400 and 200 mg/kg) significantly decreased AST, ALT and ALP level, whereas WEA 100 mg/kg non-significantly decreased these levels when compared with arthritic control group **Table 2**.

The level of TP was significantly decreased in the arthritic control group when compared to the healthy control group. Etoricoxib and WEA (200 and 400 mg/kg) were found to be significant in restoring it when compared to the arthritic control group. However, WEA 100 mg/kg non-significantly restored the TP level **Table 2**.

Effect of WEA on Serum Cytokine Levels in FCA Induced Arthritis in Rats: The challenge with FCA caused a significant increase in serum TNF-α, IL-1β and IL-6 levels, when compared to a healthy control group. Treatment with WEA(200 and 400 mg/kg) caused a significant decrease in the serum TNF-α, IL-1β, and IL-6 levels as compared to arthritic control group **Table 2**.

TABLE 1: EFFECT OF WEA ON HEMATOLOGICAL PARAMETERS IN FCA INDUCED ARTHRITIS IN RATS

Groups	RBC (10^3cells/mm^3)	WBC (10^3cells/mm^3)	Hb (gm/dL)	Platelet (10^3cells/mm^3)	ESR (mm/h)	CRP (mg/lit)
Healthy Control	6.932 ±0.105	7.625 ±0.211	14.48 ±0.240	9.157 ±0.112	8.350 ±0.189	1.592 ±0.033
Arthritic Control	3.312 ±0.065$^{\#}$	15.39 ±0.255$^{\#}$	8.782 ±0.151$^{\#}$	18.47 ±0.274$^{\#}$	15.40 ±0.339$^{\#}$	6.928 ±0.229$^{\#}$
Etoricoxib 10 mg/kg	5.953 ±0.090***	12.85 ±0.451***	13.22 ±0.128***	13.95 ±0.245***	9.850 ±0.375***	3.255 ±0.088***
WEA 100 mg/kg	3.183 ±0.043	15.33 ±0.058	9.050 ±0.134	18.04 ±0.076	14.17 ±0.125*	6.417 ±0.207
WEA 200 mg/kg	4.197 ±0.212**	13.75 ±0.421**	9.892 ±0.384**	17.28 ±0.200**	13.63 ±0.305**	5.948 ±0.237**
WEA 400 mg/kg	5.042 ±0.272***	13 ±0.128***	13.20 ±0.224***	15.24 ±0.293***	11.57 ±0.416***	4.902 ±0.086***

WEA: wedelolactone rich extract of *Eclipta alba*; FCA: Freund's complete adjuvant, RBC: red blood cell, WBC: white blood cell, Hb: hemoglobin, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein. Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with arthritic control group and $^{\#}P<0.001$ when arthritic control group compared with healthy control group.

TABLE 2: EFFECT OF WEA ON BIOCHEMICAL PARAMETERS AND CYTOKINESIN FCA INDUCED ARTHRITIS IN RATS

Groups	TP (gm/dL)	AST (U/L)	ALT (U/L)	ALP (U/L)	TNF-α(pg/ml)	IL -1β(pg/ml)	IL –6(pg/ml)
Healthy Control	7.21 ±0.144	41.81 ±1.275	53.56 ±1.156	75.07 ±1.550	38.67 ±1.022	120.5 ±0.522	140.1 ±1.104
Arthritic Control	5.583 ±0.179$^{\#}$	124.5 ±1.804$^{\#}$	186.9 ±1.433$^{\#}$	446.2 ±2.420$^{\#}$	120.2 ±1.447$^{\#}$	426.6 ±3.491$^{\#}$	407.0 ±1.936$^{\#}$

Etoricoxib 10 mg/kg	7.433 ±0.128***	109.3 ±0.997***	172.8 ±3.688***	435.1 ±1.566***	114.3 ±1.202*	413.9 ±2.230*	398.7 ±2.519*
WEA 100 mg/kg	5.767 ±0.185	117.3 ±2.321*	179.3 ±2.025*	441.8 ±1.204	111.5 ±2.172**	408.0 ±2.565**	400.7 ±1.965
WEA 200 mg/kg	6.200 ±0.093*	115.7 ±1.437**	175.0 ±1.508**	436.7 ±2.463**	107.8 ±1.424***	319.6 ±3.599***	399.7 ±1.295*
WEA 400 mg/kg	6.9 ±0.0912***	107.8 ±2.173***	169.4 ±0.870***	431.6 ±1.480***	97.33 ±1.333***	268.7 ±5.067***	394.6 ±1.957***

WEA: wedelolactone rich extract of *Eclipta alba*; FCA: Freund's complete adjuvant, TP: Total protein, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: alkaline phosphatase, TNF- α: Tumor necrosis factor- α, IL - 1β: Interleukin-1β, IL-6: Interleukin-6. Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test*P<0.05, **P<0.01, ***P<0.001 when compared with arthritic control group and #P<0.001when arthritic control group compared with healthy control group.

Effect of WEA on Spleen and Thymus Weight in FCA Induced Arthritis in Rats: There was significant increase in the spleen and thymus weight in FCA induced arthritic control animals when compared to healthy control group. Treatment with etoricoxib and WEA (400 and200 mg/kg) significantly reduced the spleen and thymus weights when compared to an arthritic control group. However, WEA 100 mg/kg did not change spleen and thymus weight when compared to arthritic control group **Table 3**.

Effect of WEA on Antioxidant Parameters in FCA Induced Arthritis in Rats: There was a significant decrease in GSH and SOD levels while significant increase in MDA levels in arthritic control group observed when compared with healthy control group.

Treatment with etoricoxib and WEA (400 and 200mg/kg) significantly increased GSH and SOD levels while significantly decreased MDA level **Table 3**.

TABLE 3: EFFECT OF ORAL ADMINISTRATION OF WEA ON SPLEEN AND THYMUS WEIGHT AND ANTIOXIDANT PARAMETERS IN FCA INDUCED ARTHRITIS IN RATS

Groups	Spleen weight (gm)	Thymus weight (gm)	SOD (mU/mg protein)	MDA (nmole MDA/mg protein)	GSH (µmol/mg protein)
Healthy Control	0.5383 ±0.010	0.09483 ±0.017	4.470 ±0.033	1.992 ±0.013	70.56 ±0.598
Arthritic Control	0.8017 ±0.012#	0.22000 ±0.005#	2.433 ±0.028#	3.437 ±0.013#	44.33 ±0.829#
Etoricoxib 10 mg/kg	0.6517 ±0.012***	0.13500 ±0.004***	2.872 ±0.028***	2.957 ±0.018***	55.84 ±0.632***
WEA 100 mg/kg	0.7600 ±0.014	0.19170 ±0.006	2.418 ±0.029	3.387 ±0.024	46.76 ±0.693
WEA 200 mg/kg	0.7350 ±0.012**	0.17170 ±0.007**	2.617 ±0.061**	3.332 ±0.019**	48.45 ±0.515**
WEA 400 mg/kg	0.6733 ±0.008***	0.15000 ±0.003***	3.177 ±0.039***	2.788 ±0.030***	52.98 ±0.939***

WEA: wedelolactone rich extract of *Eclipta alba*; FCA: Freund's complete adjuvant, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione reductase. Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test. **P<0.01, ***P<0.001 when compared with an arthritic control group and #P<0.001when arthritic control group compared with the healthy control group.

Effect of WEA on Histopathological Analysis of Ankle Joint in FCA Induced Arthritis in Rats: Histopathology of ankle joint of healthy control rats showed no signs of inflammation, with intact synovial lining **Fig. 7A**. Histopathology of ankle joint of arthritic control animals showed a massive influx of inflammatory cells, chronic inflammation and disturbed synovial lining **Fig. 7B**. In contrast to these, histopathology of ankle joint of animals treated with WEA (400mg/kg) and etoricoxib showed significant protection against synovial lining and low influx of inflammatory cells **Fig. 7F** and **Fig. 7C**, respectively. WEA (200 mg/kg) treated rats showed a moderate disturbance in the synovial lining with little presence of inflammatory cells **Fig. 7E** and WEA (100 mg/kg) treated rats showed influx of inflammatory cells with evidence of disturbed synovial lining **Fig. 7D**.

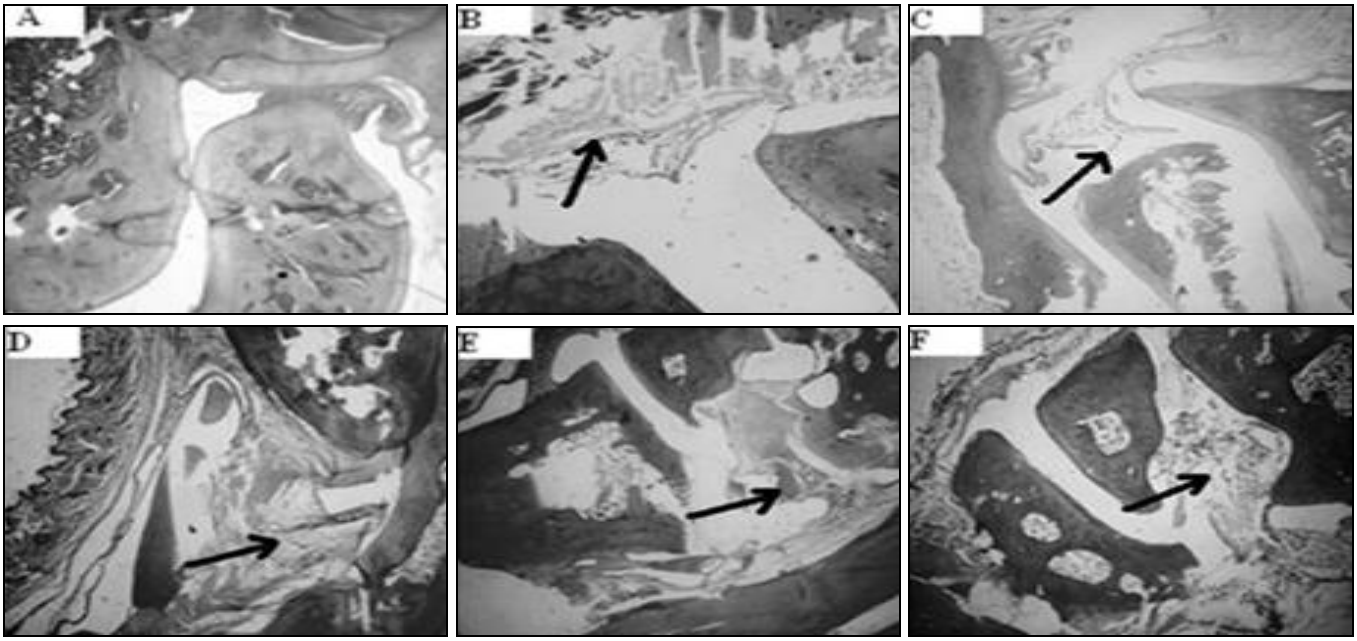

FIG. 7: HISTOPATHOLOGY OF ANKLE JOINT. 6A: Healthy control: Joint- Joint Bone with no infiltration of inflammatory cells exudate in joint tissue H&E 10X; Thickness: 5µ; Magnification: 40X 6B: Arthritic Control: Joint- Joint bone with maximum infiltration of inflammatory cells exudate in joint tissue. H&E 10X; Thickness: 5µ; Magnification: 40X 6C: Etoricoxib 10 mg/kg: Joint- Joint Bone with no infiltration of inflammatory cells only minimal exudate in joint tissue H&E 10X; Thickness: 5µ; Magnification: 40X 6D: WEA 100mg/kg: Joint- Joint Bone with Moderate infiltration of inflammatory cells and exudate in joint H&E 10X; Thickness: 5µ; Magnification: 40X 6E: WEA 200mg/kg: Joint- Joint Bone with minimal infiltration of inflammatory cells and fibrous tissue H&E 10X; Thickness: 5µ; Magnification: 40X 6F: WEA 400mg/kg: Joint- Joint Bone with no infiltration of inflammatory cells only minimal exudate in joint tissue H&E 10X; Thickness: 5µ; Magnification: 40X.

DISCUSSION: RA is a chronic autoimmune inflammatory disease. The existing drugs relieve pain and inflammation without much effect on disease progression. The use of biological is limited, owing to the cost involved. Herbs are promising, inexpensive, highly tolerated, and are vital source of new therapeutic agents for various diseases. Employing herbal medicines based on their traditional use for the treatment of chronic diseases has increased in the last few decades. The various bioactive phytoconstituents present in plants are more effective as they act on multiple sites and shows synergistic activity [22, 23]. *Eclipta alba*is traditionally claimed to alleviate pain and inflammation. Wedelolactone is a major constituent of *Eclipta alba* reported to inhibit cytokines and to possess anti-inflammatory and immunomodulatory effect. Considering all these reported activities, we evaluated the antiarthritic potential of wedelolactone rich fractions of *Eclipta alba* in FCA induced arthritis in rats.

Enrichment of active phytoconstituent leads to potentiation of pharmacological activity; hence in the present study we prepared enriched extract of wedelolactone. The content of wedelolactone in the ethanolic and enriched fraction was quantified by a validated HPLC method. The enrichment was significantly high, with a 4-fold increase in the content of wedelolactone when compared to ethanolic extract.

FCA induced arthritis is a type of cell-mediated immunity that results in synovial inflammation characterized by an accumulation of T cells, plasma cells, macrophages, increased numbers of blood vessels, and hyperplasia of the invasive intimal lining with intense immunological activity in synovial environment [24, 25]. The present study demonstrated that treatment with WEA dose-dependently attenuated adjuvant-induced arthritis and facilitated recovery, as shown by a decrease in paw volume, hyperalgesia, cytokine levels, which are further substantiated by histopathology of ankle joints. Bodyweight is considered an indirect index of health status and disease recovery.

Proinflammatory cytokines cause acute increase in leptin levels leading to weight loss by anorexia and elevated metabolic rate [26]. WEA prevented the weight loss indicating their ability to suppress these proinflammatory cytokines.

In arthritis increase in paw, volume is due to inflammation caused by synovial destruction mediated through proinflammatory cytokines [25]. In the present study, WEA significantly inhibited the inflammation, which is evident by decreased paw volume.

Inflammation causes hyperalgesia, increased responses to noxious stimuli, which are mediated by release of prostaglandins and other endogenous mediators [27]. This hyperalgesia limits the ability of the patient to do daily activities and therefore diminishes the quality of life.

In the present study, the pain threshold in arthritic control group significantly decreased while WEA treatment increased pain threshold in mechanical hyperalgesia, thermal hyperalgesia and tactile allodynia screening. This confirms the anti-inflammatory and analgesic potential of WEA.

RA causes a decrease in RBC and Hb while increase in ESR as the presence of proinflammatory cytokines decreases iron absorption and transport, decreases erythropoiesis and causes premature destruction of RBCs while IL-1 stimulates a moderate rise in WBCs [28]. WEA significantly increased RBCs count and Hb level while decreased ESR and WBC count. These findings confirmed the attenuation of inflammatory cytokines. Serum AST and ALT play important role in the formation of inflammatory mediators and hence serves as a simple and excellent tool to measure the antiarthritic activity.

ALP levels are increased in case of bone erosion and periarticular osteopenia, a sensitive marker of cellular integrity and cellular toxicity induced by pathological conditions [29]. Treatment with WEA caused decrease in AST, ALT and ALP levels which confirmed the decrease in inflammation and bone erosion.

TNF- α and IL-6 are proven therapeutic targets (biologicals) to treat RA. Cytokines mainly TNF-α, IL-1β, and IL-6 secreted by macrophages promotes induction of adhesion molecules and proteinase gene expression, which in turn plays an important role in joint destruction. TNF- α mainly causes inflammation while IL-1 is responsible for cartilage and bone destruction, induction of acute-phase proteins by hepatocytes, stimulation of prostaglandin and collagenase production by synovial cell [26].

Wedelolactone has been reported to have anti-inflammatory action by inhibiting the release of cytokines, especially IL-1 β. The observed decrease in the disease severity was associated with reduced levels of inflammatory cytokines TNF- α, IL-1 β and IL-6 when treated with WEA owing to the presence of wedelolactone.

Free radicals generated during RA causes phagocytosis of immune complexes leading to lipid peroxidation which increased MDA content, decreased GSH and SOD levels in arthritic animals as a consequence of their consumption during oxidative stress and cellular lysis [30]. In the present study, WEA administration reversed these effects, which validated the antiarthritic potential of WEA.

Autoimmunity in arthritis leads to splenomegaly generalized lymphadenopathy and altered hepatic function [31]. Spleen and thymus weight was significantly increased in arthritic control group as compared to healthy control group, while WEA decreased spleen and thymus weight. This indicated the inhibition of lymphocytes and decreased immunological response on WEA treatment.

Histological changes of ankle joint revealed the elevated number of inflammatory cells, chronic inflammation and disturbed synovial lining which are hallmarks of RA was found in arthritic control animals. WEA treatment was able to reverse the histological findings to normal. This effect of WEA on joints cartilage in arthritic rats is mediated by attenuation of inflammatory cytokines and antioxidant activity.

CONCLUSION: WEA was found to possess considerable antiarthritic potency as evident by alleviated paw volume, cytokine levels and other critical parameters associated with arthritis. This is attributed to the presence of wedelolactone a proven antiinflammatory agent along with other phytoconstituents responsible for its effectiveness.

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